Experimental use of FEMALE SPERM in the Production of Monosex Female Stocks of Chinook Salmon *(Oncorhynchus tshawytscha)* at Commercial Fish Farms

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EXPERIMENTAL USE OF "FEMALE SPERM" IN THE PRODUCTION OF MONOSEX FEMALE STOCKS OF CHINOOK SALMON (*Oncorhynchus tshawytscha*) AT COMMERCIAL FISH FARMS

by

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ABSTRACT

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The control of sex differentiation in salmonids, with the purpose of producing sterile or all-female stocks, is one of the major goals in the commercial mariculture of Pacific salmon. Experimental batches of milt,containing only female spermatozoa obtained by hormonal masculinization of genotypic chinook salmon females, have been made available to the mariculture industry in British Columbia since 1983.

Twenty-one phenotypic males, having a female genotype, were used in the experiment in the fall of 1985. The sperm, collected by repeated manual stripping over a period of 25 days, was used to fertilize normal ova from sea-run and captive chinook broodstock at 7 salmon farms and at the Capilano River Hatchery.

Quantitative and statistical analysis of sperm production included frequency of milt collection, volume of milt produced, periodic determination of spermatocrit and subjective evaluation of sperm motility. The fertility of the sperm was determined by survival of eggs to the eyed stage.

Fertilization rates were variable, ranging from 16 to 76% at the fish farms and up to 81% at the Capilano Hatchery.

Close to 550 thousand monosex female chinook alevins were produced of which about 90% are being reared at the commercial facilities. The remainder are part of the production of the Capilano River Federal Salmon Hatchery and will be released as part of an ongoing research program on the enhancement of depleted chinook stocks.

The effect of repeated stripping, sperm transport and storage conditions, origin and quality of the gametes, and suggestions for improvement of the procedures to maximize the efficiency of the technique are discussed.

Key words: chinook salmon, Oncorhynchus tshawytscha, mariculture, sperm, fertilization, survival

RÉSUMÉ

Solar, I. I., I. J. Baker, E. M. Donaldson. 1987. Experimental Use of "Female Sperm" in the Production of Monosex Female Stocks of Chinook Salmon (Oncorhynchus tshawytscha) at commercial fish farms. Can. Tech. Rep. Fish. Aquat. Sci. No. 1552: 14 p.

Un des principaux buts de la mariculture commerciale des saumons du Pacifique est le contrôle de la différenciation sexuelle chez les salmonidés afin de produire des stocks stériles ou entièrement femelles. Depuis 1983, l'industrie marécole de la Colombie-Britannique dispose de lots expérimentaux de laitance ne contenant que des spermatozoides femelles. Cette laitance a été obtenue par masculinisation hormonale de saumons quinnats de génotype femelle.

L'expérience menée à l'automne 1985 portait sur 21 mâles phénotypiques à génotype femelle. On s'est servi de la laitance, recueillie par extraction manuelle répétée pendant 25 d, pour fertiliser des oeufs normaux obtenus de géniteurs sauvages et d'élevage gardés à sept salmonicultures et à la piscifacture de la rivière Capilano.

Les analyses quantitatives et statistiques de la production de sperme portent sur la fréquence d'extraction de la laitance, le volume de laitance recueilli, la détermination périodique du spermatocrite et l'évaluation subjective de la motilité du sperme. La survie des oeufs jusqu'au stade embryonné a servi d'indicateur de la fertilité du sperme.

Les taux de fertilisation étaient variables: ils allaient de 16 à 76 % aux salmonicultures et atteignaient 81 % à la piscifacture de Capilano.

On a produit presque 550 000 alevins quinnats femelles unisexes, dont environ 90 % sont actuellement élevés dans les établissements commerciaux. Le reste fait partie de la production de la piscifacture fédérale de la rivière Capilano; ces quinnats seront relâchés dans le cadre d'un programme premanent de recherche sur la mise en valeur des stocks de saumon appauvris.

On trait aussi de l'incidence de l'extraction répétée, des conditions de transport et d'entreposage du sperme et de l'origine et de la qualité des gamètes. Des suggestions pour la mise au point des méthodes afin de maximiser l'efficacité de la technique sont aussi présentées.

Mots-clés: saumon quinnat, Oncorhynchus tshawytscha, mariculture, sperme, fertilisation, survie

INTRODUCTION

The rapid development of salmonid mariculture in the Pacific Northwest during the past decade has resulted in an increased interest in the control of sex in chinook and coho salmon, particularly in the application of techniques to manipulate the sex ratios and to reduce or eliminate the potential for sexual maturation in salmonids.

Research conducted at the West Vancouver Laboratory, (WVL), Biological Sciences Branch, Dept. of Fisheries and Oceans, has resulted in the development of a number of hormonal and chromosome set manipulation techniques oriented to increase the proportion of female salmon or to the production of sterile stocks (Donaldson and Hunter, 1982).

One of these techniques involves a two stage androgen treatment to produce genotypic females having a male phenotype the sperm of which, when used to fertilize normal ova, produces an all-female stock (Hunter et al. 1983).

The objectives and management applications of monosex female stocks in the intensive mariculture or sea ranching of salmonids have been discussed by Hunter and Donaldson (1983) and Donaldson and Hunter (1985). The application of the technique by itself or in combination with other biotechnologies, such as triploid production, has been reviewed by Donaldson (1986).

In order to transfer the technology to the growing mariculture industry in British Columbia, initial quantities of "female sperm" or "all-female" eyed eggs have been made available to commercial fish farms since 1983. At the same time, in an effort to contribute to the enhancement of depleted Capilano River chinook salmon stocks, externally marked, all female groups have been released from the Capilano Salmon Hatchery. In addition, externally marked masculinized groups (50% male:50% female or 100% female genotype) have been released from the hatchery for the purpose of providing all female or 75% female milt on their return.

In this report we describe the experimental use of female spermatozoa, obtained from captive chinook broodstock reared at the West Vancouver Laboratory, in the production of monosex female stocks at 7 mariculture facilities and the Capilano Hatchery in 1985.

MATERIALS AND METHODS

EXPERIMENTAL FISH

The male broodstock consisted of 21, four and five year old chinook

salmon produced in 1980 and 1981 by sex inversion of recently hatched female alevins by androgen treatment. The fish were reared in 3m diameter fiberglass tanks at the West Vancouver Laboratory. About a month prior to spawning the fish were individually identified with numbered anchor tags (Floyd Tag & Mfg., Seattle, Wa.), and gradually acclimated to fresh water.

The ova were collected at the fish farms (Table 1) from net-pen reared female broodstock or at the Big Qualicum, Robertson Creek or Capilano River Hatcheries.

The males were repeatedly stripped by manual abdominal pressure over a period of 25 days (October 17 to November 12, 1985). The sperm was collected in graduated beakers and transferred into 18 oz. whirl-pack bags in batches of 10 to 30 ml. The bags were marked with the male identification number, filled with oxygen, sealed, then stored in coolers over crushed ice and transported to the fish farms. The time from stripping of the sperm to fertilization of the ova ranged from 2 h (Capilano Hatchery) to as long as 12 h, when the sperm was used at fish farms situated in remote locations.

INDUCED SPERMIATION

Hormonal treatment was used to stimulate spermiation in fish that were not producing sperm at the beginning of the second week of stripping. Five males were injected on Oct. 24 with a gonadotropin-releasing hormone analogue (GnRHa, D-Ala^{6}-des-gly^{10}LH-RH ethylamide, Syndel Laboratories, Vancouver, B.C.). The hormone solution, 0.02 mg/kg body wt in 0.4 ml/kg body wt 0.7% saline, was injected at a shallow angle at a point just posterior to the pelvic fin using a syringe with 21G x 1.25 needle (Becton, Dickinson and Co., Rutherford, N.J., USA).

SPERMATOCRIT AND SPERM MOTILITY

Subsamples of sperm were taken periodically from individual fish prior to bagging, for motility tests using a 160X standard microscope and 0.7% saline. Subjective evaluation of the quantity of motile cells and duration of activity was performed using a scale of 1 to 5. Samples were also taken for determination of spermatocrit index (% of packed cell in total milt volume). The volume of milt produced by each fish at each stripping was recorded.

FERTILIZATION TESTS

Single male/female crosses were conducted at the Capilano Hatchery on October 23 using 15 of the 21 males. Additionally, tests using different ratios of sperm to ova (1, 5 and 15 ml/l) were carried out at the Laboratory

(WVL) on October 31 using milt from 3 random males stored overnight in conditions similar to those used to transport the sperm to the fish farms. Fertilization rates were determined by observation of embryo development in samples of 20 to 30 eggs cleared in a 5% aqueous solution of acetic acid.

Survival rates at the fish farms and Capilano Hatchery were determined at the eyed-egg stage. Sex ratio of the progenies of the 15 males used at the Capilano Hatchery were determined at seven months post-hatch by dissection of 20 fish from each crossing and visual observation of the gonads.

HEALTH CHECKS

At the end of the spawning period the males were terminated and measured for weight and length. Frozen (early mortalities) and fresh carcasses were sent to the Diagnostic Service, Fish Health Section, Pacific Biological Station, for health inspection.

STATISTICAL ANALYSIS

Means and standard deviation (S.D.) were calculated of individual fish weights and lengths, and of spermatocrit readings (4 to 7 observations per fish).

Correlation analysis were conducted to test possible relationships of size of the fish and total volume of sperm produced, the % spermatocrit and the fertilizing ability of the sperm, the effect of repeated stripping and the percent fertilizations achieved at Capilano and one of the fish farms (Facility G, Table 5) and between the volume of sperm used (ratio of sperm to eggs in the range of 1-15 ml/l) and the percent fertilization achieved in domestic broodstock eggs at the laboratory.

RESULTS

Sperm Production and performance

The male broodstock (year classes combined), ranging in size from 1.6 to 6.3 kg (mean 3.8 ± 1.5 kg), were stripped of sperm from 4 to 10 times over a period of 25 days. (Table 2). The hormonal treatment did stimulate spermiation in the 5 fish injected. The total volume of sperm collected from the 21 males was close to 4.6 l. Individual sperm production was highly variable ranging from 65 ml (4 strippings, 4.5 kg fish, hormone induced) to 420 ml (10 strippings, 4.2 kg fish, spermiation not induced). Mean volume of sperm collected per kg body wt was 76.4 + 49.5 ml.(range 14.4 - 204.4 ml/kg).

No correlation was observed between the size of the fish and the total volume of sperm produced (r = 0.1)

Percent spermatocrit also showed a wide range of variation (Table 3). Overall mean spermatocrit was 21.9 ± 7.4% (range 10.9 - 39.7%). Correlation analysis showed that there was no relationship between the percent spermatocrit and the fertilizing ability of the sperm. (r = 0.24). Similarily, the correlation coefficient of the regression analysis between frequency of stripping and percentage fertilization achieved did not suggest a relationship between the two variables. (r = 0.2).

The fertilization test conducted in the laboratory using sperm from 3 males in three ratios of sperm to ova (1, 5 and 15 ml/1), after storage for 16 h, produced fertilizations similar to or lower than the respective controls.(Table IV). However, no effect of the volume of sperm used was evident in the percent fertilizations observed.

Fertilization rates and sex ratio of progenies

Fertilization rates at the fish farms determined as percent survival to the eyed-egg stage ranged from 15.8 to 76.1% (Table 5). The total adjusted mean fertilization rate (to account for different quantities of eggs fertilized at each location) was 53.8%. Mean fertilization rate at the Capilano Hatchery was 80.1%. All 20 fish sampled from each family turned out to be female. As a result, from a total of 994 thousand eggs from pen-reared and sea-run chinook females approximately 554 thousand all-female alevins were produced. Of these, close to 90%, (496 thousand) will be reared to market size at commercial fish farms. The remainder, were released from the Capilano Hatchery in June 1986, as in previous years since 1983, in an effort to increase the proportion of females in future hatchery escapements.

Health condition

All 21 male broodstock inspected at the Diagnostic Service, PBS, were found free of pathogens such as Renibacterium salmoninarum (BKD) and the viral agent causative of Infectious Hematopoietic Necrosis (IHN).

DISCUSSION

Despite the somewhat low fertilization rates achieved at the fish farms, more monosex female chinook smolts were transferred to seawater in 1986 than in any previous year since the experimental program started in 1983 (M. Coon, 1986 pers. comm.).

The variability of the results at the different hatcheries suggests that a number of factors may have played a role in the reduced fertilization rates observed. The time elapsed between stripping of the sperm and

insemination of the eggs, variable storage and transport conditions, and insemination procedures, may have contributed to a reduction in the fertilizing potential of the sperm. We examined the frequency of stripping as a possible factor of reduced sperm performance. Our analysis of the available data showed that the fertility of sperm batches collected from fish being stripped for the sixth time was not significantly different from those batches obtained in earlier strippings.

Eight of the 15 test fertilizations conducted at the Capilano Hatchery produced percentage survival rates to the eyed-egg stage above 90%. One cross, however, resulted in 9.3% survival. The mean percentage fertilization rate of 15 crosses (80.1%), although lower than the mean of 5 control crosses (90.7%) was not significantly different and could be deemed satisfactory. At the Chapman Creek Hatchery (Cockburn Bay Sea Farms) the sperm from every fish but one was used at least once in three different egg takes with a combined mean fertilization of 76% (B. Swift, 1986, pers. comm.). The generally satisfactory results obtained at Capilano Hatchery and Chapman Creek Hatchery make the low fertilization rates observed at other locations (15 to 50%) difficult to explain.

Concurring with the notion of overall reduced fertilization ability of the sperm in 1985 compared to previous years (91 and 96%, in 1983 and 1984, respectively, at Capilano Hatchery) we are inclined to attribute the somewhat poorer results of 1985 to the interaction of 2 factors:

a) intrinsic reduced quality of the gametes probably caused by the long term captivity of the broodstock fish under less than optimal rearing conditions and feeding regimes.

b) reduced motility of the sperm (intensity and duration) after several hours of storage and transport prior to use at the fish farms.

In consideration of the results obtained in 1985, with the objective of maximizing the fertilizing potential of the sperm during the 1986 spawning season, a detailed set of instructions was prepared for the use of the chinook salmon monosex female sperm. This document (Appendix A), was attached with every shipment of sperm sent to the fish farms in the fall of 1986.

It is our belief, however, that more significant gains are to be made by substantial improvement of broodstock rearing conditions and by the development of specially designed broodstock diets. Additionally, in accordance with the main objective of the technology transfer process, we would expect that most of the fish farms that received the sperm will take advantage of the lead time gained and develop their own "on site" source of female sperm (masculinization of genotypic females), thus eliminating the second possible cause of reduced fertilization: storage and transport. This, however, should be considered as a short-term solution. The ultimate goal should be the development of an "all-female" line to which chinook salmon from diverse genetic backgrounds and superior domestic traits should be incorporated.

Furthermore, with the development of complementary chromosome set manipulation techniques, alternative means of producing screened genotypic females (gynogenesis) for masculinization and "female sperm" production will

become available to the fish farmer in the near future (Donaldson, 1986). The application of heat or hydrostatic pressure shock to all-female eggs shortly after fertilization may enable the commercial aquaculturists to produce all-female triploids which, being effectively sterile, may provide a further option for production purposes (Lincoln and Scott, 1983; T. Benfey, in preparation).

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Table 1. Experimental use of "Female Sperm" at Commercial Mariculture Facilities - 1985 - Participating Fish Farms

- Alberni Marine Farms Ltd.	Port Alberni
- Cockburn Bay Sea Farms Ltd. Chapman Creek Hatchery	Sechelt
- Tidal Rush Marine Farms Ltd. Hotham Sound Hatchery	Sechelt
- Sea Spring Salmon Farm Ltd.	Chemainus
- Seastar Resources Corp.	Sechelt
- Suncoast Salmon Ltd.	Sechelt
- Western Aquaculture Res. Inc.	Port Alberni
Also:	
- Capilano Salmon Hatchery	North Vancouver

Table 2. Experimental Use of Chinook Salmon "Female Sperm" at Commercial Mariculture Facilities and Capilano Salmon Hatchery - 1985 - Sperm Production Data

Number of Fish Used	21
Mean Weight (n=16)	3.8 ± 1.5 K (range: 1.6 - 6.3 Kg)
Period Sperm Collection	Oct. 17 - Nov. 12 (25 days)
Number of Times Sperm Collected	4 - 10
Total Vol. Sperm Collected	4,597 ml
Mean Vol. Sperm Produced:	
Per Fish	218.9 ± 102.8 ml (r = 65 - 420)
Per Stripping	27.0 ± 11.1 ml
Per kg Body Wt	76.4 ± 49.5 ml (r = 14.4 - 204.4)

Table 3. Experimental use of chinook salmon female sperm at commercial mariculture facilities and Capilano Salmon Hatchery - 1985 - Sperm performance data

Fish #	N stripped	Spermatocrit X %		Fertilization[1] X %	
1	9	33.5		87.9	
2	9	39.7	(Highest)	75.4	
3	10	13.6		70.8	
4	8	23.2		69.0	
5	8	19.6		81.0	
6+	6	16.5		90.1	
7+	6	23.0		83.8	
8	8	30.2		91.1	
9	10	19.5		77.4	
10+	5	30.3		87.6	
11	9	29.2		95.0	(Highest)
12	8	25.0		49.4	
13	8	21.0		79.1	
14	9	21.7		64.0	
15+	4	25.0		93.3	
16	5	12.9		87.1	
17	9	10.9	(Lowest)	68.6	
18	7	14.1		28.2	(Lowest)
19+	6	13.4		92.3	
20	8	15.5		81.7	
21	9	22.2		91.0	

\+ Hormone induced
[1] Data from Capilano Hatchery and Chapman Creek Hatchery
Dates: 19-23-28-30/October 1985

Table 4. Percent fertilization[1] of domestic chinook salmon eggs using stored[2] "female sperm" in different ratios of sperm volume to ova, relative to controls.

Male[3]	Sperm Volume ML/L Eggs[4]		
	1	5	15
A	60.9	67.3	66.9
B	86.1	100.0	81.6
C	74.5	78.3	92.8

Notes
1 10 day-old embryo development
2 16 hrs storage in sealed plastic bags with oxygen, on ice
3 3 males ramdomly selected
4 Pooled eggs from domestic broodstock (Nanaimo PBS and Tidal Rush).

Table 5. Experimental use of Chinook Salmon "Female Sperm" at Commercial Mariculture Facilities and Capilano Salmon Hatchery - 1985 - Monosex stocks production data.

Salmon Farm	Vol. Sperm Used (ML)	%	Quantity of Eggs Fertilized 000's	Survival to Eyed Stage 000,s	%
A	101	2.4	22.2	3.5	15.8
B	819	19.1	301.8	125.8	41.7
C	181	4.2	125.0	74.3	59.4
D	1622	37.8	105.8	24.7	23.3
E	51	1.2	27.0	11.0	40.7
F	51	1.2	10.0	4.8	48.0
G	1239	28.9	331.2	252.2	76.1
Sub Total	4064	94.8	923.0	496.3	53.8
Capilano Hatchery	225	5.2	71.4	57.2	80.1
Grand Total	4289	100.0	994.4	553.5	55.7

APPENDIX A

Instructions for use of chinook salmon "female sperm"

Dept. Fisheries and Oceans, Fisheries Research Branch,
West Vancouver Laboratory
October 1986
Prepared by I.I. Solar

1. Sperm collection and storage (at West Vancouver)
   - The sperm will be collected from individually identified males and stored in 170 ml (6 oz) whirlpack bags, with oxygen. Only 5 ml (0.18 oz) of sperm will be placed in each bag for a 1:34 ratio sperm to oxygen. The bags, marked with the ID number of the donor fish, will be placed laying horizontally in a cooler on a bed of crushed ice.

2. Egg collection (at fish farms)
   - We recommend that the gametes be collected with utmost care avoiding contamination with blood and feces. Crushing of eggs should also be avoided to reduce or prevent the occurrence of broken eggs. Ovarian fluid should not be drained. To test for presence of egg protein in the ovarian fluid, which reduces sperm motility and thereby fertility, sample about a teaspoon of fluid and add freshwater. If the sample turns a cloudy white colour conduct the following procedure:

   a) Drain the ovarian fluid from the eggs.
   b) Rinse the eggs twice in double their volume of a 1.4% solution of $NaHCO_3$ (14 g sodium bicarbonate per litre distilled water at 7-8°C).
   c) Decant the solution and add 1/4 the egg volume of fresh sodium bicarbonate solution (example: 250 ml solution per litre of eggs).
   d) Inseminate the eggs immediately.

3. Transport

   The sperm should be transported in the tightly closed coolers and used to fertilize the eggs within six hours. Temperature of the sperm, during transport, should be between 1 and 4°C.

4. Motility test

For a subjective test of sperm motility, as an indicator of fertilizing ability, the following materials are required:

- Standard microscope with 160X minimum magnification.
- Microscope glass slides (e.g., 75 x 25 mm).
- Microscope cover slips (e.g., 22 x 40 mm).
- Plastic or glass disposable pipettes.
- 0.7% saline solution (7 g NaCl per litre distilled water)

a) Place a small drop of semen on the glass slide.

b) Cover with the glass cover slip.

c) Introduce a small drop of the saline solution under the cover slip.

d) Observe immediately evaluating multidirectional movement and proportion of motile sperm cells. Motility of 70-100% for 30 seconds is acceptable. Discard sperm batches showing less than 40% motile cells for less than 10 seconds.

5. Fertilization

a) Place the eggs in a plastic container with rounded bottom and smooth surface and rim.

b) Inseminate by pouring sperm over eggs at a rate of no less than 3 ml per litre.

c) Use sperm from only one male at a time. Record ID number.

d) Mix sperm and eggs by hand (short, smooth fingernails) tilting the container to ensure a complete mixing.

e) Let stand in a cool place, covered to protect from sunlight and water contamination, for about 5 minutes.

f) Rinse with 0.5 litre of clean hatchery water per liter of eggs.

g) Transfer to disinfection bath.

6. Disinfection

After rinsing, the fertilized eggs should be handled with extreme care avoiding all mechanical shock. Disinfection may be carried out in the incubator tray in the Heath stack by static bath in a solution of bridine (1% active iodine). Apply the disinfection treatment as follows:

a) Add 110 ml of bridine to the incubation tray (cap. 11 litres).

b) Add 0.6 g of sodium bicarbonate.

c) Mix well.

d) Place the eggs in the bridine solution for 10 minutes.

e) Resume continuous flow.

7. Fertility check

a) Fertilization rates may be determined at the 4 cell stage by taking a sample of 10 to 20 eggs at the proper timing depending on the incubation temperature.

4°C	30 hrs
6°C	23
8°C	17
10°C	13.5

b) Place the egg sample in a 25-50 ml plastic vial.

c) Add 20-30 ml of a solution of 4% acetic acid + 0.9% sodium chloride (40 ml acetic acid + 9 g NaCl per litre distilled water).

d) Label vial properly indicating male ID number, name of facility, contact and telephone number.

e) Send samples to Frank Velsen, Pacific Biological Station, Dept. Fisheries and Oceans, Biological Sciences Branch, Nanaimo, B.C.

For further information please contact:

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